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# A New Furunculosis Challenge Method for Evaluation of Vaccine Efficacy in Rainbow Trout

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## Abstract

Experimental infection of fish for vaccine efficacy studies is associated with several limitations. Administration of live bacteria with the purpose of causing disease in fish can be performed by co-habitation, immersion or injection. We have developed a new *Aeromonas salmonicida* challenge method for rainbow trout and have applied it for evaluation of furunculosis vaccine efficacy. The method reveals development of systemic immunity in fish as live bacteria are introduced in the tail fin epidermis distant from the vaccine injection site (peritoneal cavity). This method seeks to mimic natural infection in fish farms where tail biting and therefore bacterial exposure to tail fin ulcers is widespread. By use of a multi-needle device ten epidermal perforations were introduced in the dorsal part of the tail fin of anaesthetized rainbow trout (vaccinated or naive). Subsequently 100  $\mu$ L ( $3.4 \times 10^8$  colony-forming units (CFU)  $\text{mL}^{-1}$ ) of a 48 hour culture of *Aeromonas salmonicida* subsp. *salmonicida* strain 090710-1/23 was placed at the perforation site for 60 sec whereafter fish were allowed to regain consciousness in clean freshwater. Immunohistochemistry and scanning electron microscopy illustrated the spread of bacteria from the injection site. Classical furunculosis symptoms associated with a high morbidity rate were observed in control fish whereas vaccinated fish exhibited a significantly higher survival.

## Keywords

*Aeromonas salmonicida*, Challenge Method, Furunculosis, Immunization, Salmonids

## 1. Introduction

Immunoprophylactic measures with focus on vaccination represent the most sustainable

disease preventing management tools which can be applied in fish aquaculture [1]. Vaccines against bacterial pathogens have until now shown the highest success rate but research and development efforts in the field are still highly needed. The number of teleost species being domesticated is increasing and it is expected that the number of associated diseases will rise correspondingly which will emphasize the need for development of new vaccines. In addition, there is still room for improvement of existing vaccines in cases where the protection is suboptimal [2]. In all cases reliable methods for vaccine efficacy testing are needed and therefore reproducible challenge models must be available.

Rainbow trout reared in marine net pens in Danish waters experience morbidity and mortality due to furunculosis during the summer period-despite previous vaccination against the causative pathogen *Aeromonas salmonicida* subsp. *salmonicida* (hereafter AS) [3] [4]. Therefore, one of the most urgent needs for Danish mariculture is the availability of a protective vaccine. In order to assist vaccine development initiatives we have established a new challenge method which can demonstrate a systemic response of vaccinated rainbow trout.

An optimal challenge model for testing vaccine efficacies should closely resemble the natural infection and present pathognomonic features. The existing challenge methods comprise waterborne challenge or direct injection. The waterborne challenge methods (co-habitation or immersion) closely resemble the natural exposure, but may have low reproducibility and infectivity [5] [6]. The injection challenge methods including intraperitoneal (ip.) and intramuscular (im.) injection allow direct delivery of precise dosage of live bacteria to each fish [7]. It provides superior reproducibility compared to other methods [8] but local inflammatory reactions at the vaccine injection site (body cavity) after ip. injection may rapidly eliminate injected bacteria and thereby confound estimation of the systemic protection induced by vaccination [6] [8] [9]. In addition, direct injection of bacteria does not reflect a natural infection situation where bacteria penetrate the fish surface [5] [10].

We have developed and tested a new challenge method that aims to mimic the natural infection route in aquaculture settings [11]. This method takes into consideration that fish in aquaculture are prone to physical injuries due to fin biting [12] [13] [14]. By making small perforations in the tail fin epidermis of rainbow trout we intend to imitate the injuries that fish acquire in aquaculture. We show that subsequent exposure to live virulent AS bacteria elicit furunculosis.

## 2. Materials and Methods

### 2.1. Fish and Rearing Conditions

Disinfected eyed rainbow trout eggs originating from Fousing Trout Farm, Jutland, Denmark, were hatched in a pathogen-free rearing facility at Bornholm Salmon Hatchery (Aqua Baltic, Nexø, Denmark). The maintenance of pathogen-free status has previously been described [15]. Groups of fish were vaccinated or sham-vaccinated and reared for 16 weeks at 10°C in 700 L tanks with re-circulated municipal water and fed

1% biomass daily with dry pellet feed (Bio Mar A/S, Denmark). Fish were then transported to the fish keeping facility at the University of Copenhagen, Frederiksberg, Denmark, where they were acclimatized to 19°C by raising the water temperature over 7 days before bacterial exposure. The challenge trial was conducted at 19°C in order to mimic the water temperatures reached in mariculture net pens during summer in Denmark when furunculosis outbreaks occur. The studies were approved under the license no. 2013-15-2934-00768 issued by the Animal Experiments Inspectorate, Ministry of Environment and Food, Denmark.

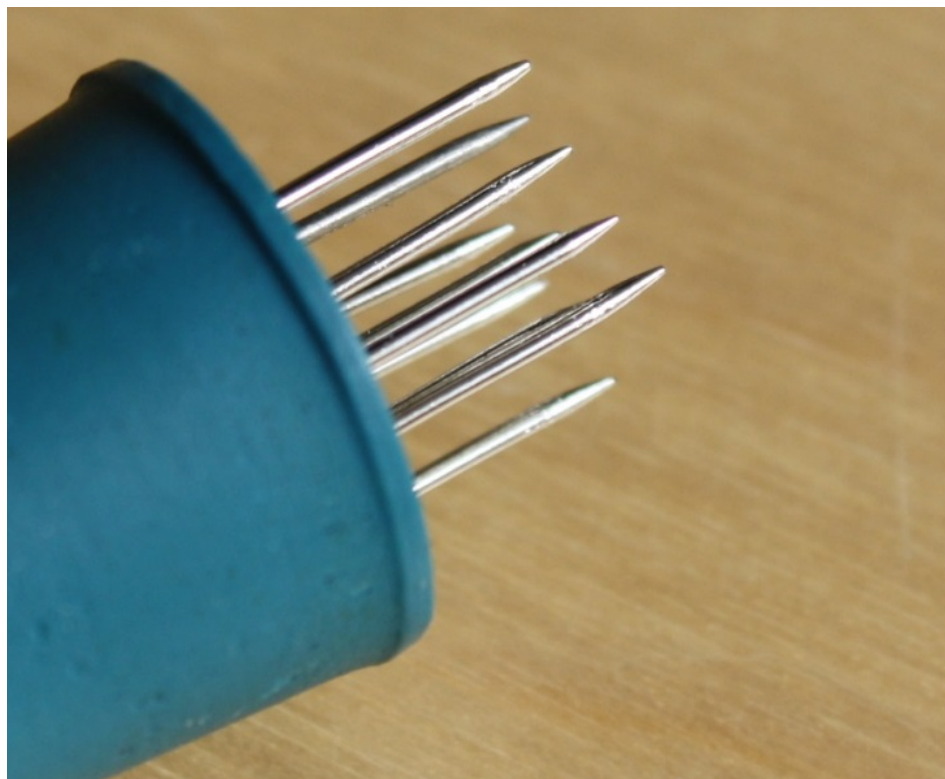
## 2.2. Vaccination

A total of 160 rainbow trout (20 - 25 g) were randomly divided into 2 groups (80 fish per group) and further subdivided into duplicate tanks each containing 40 fish. The groups comprised of 2 tanks of vaccinated fish and 2 tanks of saline control fish. All the fish were anaesthetized (75 mg·L<sup>-1</sup> MS-222, Sigma Aldrich, Denmark) and ip.-injected with 0.1 mL of either experimental vaccine or saline (0.9% NaCl) respectively. The experimental vaccine contained formalin-killed bacteria of both *A. salmonicida* subsp. *salmonicida* (AS) 090710-1/23 ( $1 \times 10^9$  colony-forming units (CFU) mL<sup>-1</sup>) and *V. anguillarum* serotypes O1 and O2a (both  $5 \times 10^8$  CFU mL<sup>-1</sup>) adjuvanted with paraffin oil. The bacteria for the experiment (vaccine and challenge) were obtained from the National Veterinary Institute of the Technical University of Denmark and previously isolated from disease outbreaks at Danish rainbow trout sea farms [3] [16]. The experimental setup also included corresponding groups of unchallenged control fish.

## 2.3. Challenge

At 17 weeks post-vaccination (wpv) 50 vaccinated fish and 50 control fish in duplicate tanks (each containing 25 fish, now with a mean weight of 45 g) were challenged with AS by a new challenge method using a multi-puncture device [11]. The multi-puncture device was composed of a plastic cylinder equipped with a rubber plug carrying 10 steel needles (Figure 1). During the challenge process the dorsal part of the tail fin was swabbed dry with a paper towel and small perforations were created by inserting the needles through the dorsal part of the tail fin of anaesthetized fish (75 mg·L<sup>-1</sup> MS-222). Then 100 µL of a 48 hour broth culture ( $3.4 \times 10^8$  CFU mL<sup>-1</sup>) of AS strain 090710-1/23 was placed on the puncture site for 60 sec where after fish were transferred to freshwater for recovery. The bacterial culture was regularly shaken throughout the challenge procedure in order to ensure the equal distribution of bacteria in the flask.

Morbidity in fish was monitored every second hour during the following 3 weeks post-challenge (wpc). Moribund fish were immediately removed for euthanasia (immersion into 300 mg·L<sup>-1</sup> MS-222) and recorded as mortalities. The moribund state of the fish was defined as a complete loss of equilibrium and strong discoloration along with the development of furuncles and hemorrhages. Swabs from head kidney of 2 freshly euthanized fish per tank were plated onto 5% blood agar plate (SSI Diagnostica, Denmark) for bacteriological analysis. The diagnosis of AS was done according to Dalsgaard and Madsen [3].



**Figure 1.** The multi-puncture device used in challenge experiments. The device was used to make 10 small puncture holes through the dorsal part of the tail fin of anaesthetized fish where after 100  $\mu\text{L}$  of *Aeromonas salmonicida* subsp. *salmonicida* strain 090710-1/23 bacterial culture ( $3.4 \times 10^8$  CFU  $\text{mg}^{-1}$ ) was layered on the puncture site for 60 sec.

#### 2.4. Scanning Electron Microscopy (SEM)

Fin samples of the puncture site (dorsal part of the tail fin) were obtained from two infected fish at day 0, 3 and 5 post-challenge (pc) and from two uninfected fish at day 0. Samples were fixed at room temperature (rt) in Karnovsky's solution [17] modified by using 0.3 M cacodylate buffer, pH 7.3, vacuum-treated for 1 h and post-fixed for  $2 \times 20$  min in 1% Osmium tetroxide ( $\text{OsO}_4$ ) fixative in 0.1 M cacodylate buffer at rt. Following a subsequent washing step in  $\text{dH}_2\text{O}$  for 20 min, fixed samples were dehydrated in series of graded acetone (10%, 20%, 30%, 50%, 70%, 90% for 20 min each and 100% for  $2 \times 30$  min). Samples were dried in 100% hexamethyldisilazane (HMDS) for 15 min, transferred to a filter paper and allowed to dry overnight (o/n). The samples were then mounted on aluminum stubs, sputter-coated with gold-palladium in Polaron SC7640 Sputter coater (Quorum technologies, UK) and studied with SEM Quanta 200 (FEI, USA).

#### 2.5. Immunohistochemistry (IHC)

Immunohistochemistry was performed as described previously [18], modified for detecting AS. Tail fin samples were collected from two fish at day 0, 3, 4, 5, 6, 8, 12 and 21 pc. Additional spleen samples were collected from five fish at day 21 pc. All samples

were fixed in 10% neutral buffered formalin for 24 h at rt and thereafter placed in 70% EtOH and stored at 4°C until embedding. Tissue samples were dehydrated in graded EtOH series (70%, 96%, 99%), cleared in xylene, embedded in paraffin and sectioned with a Leica RM2135 microtome (Leica Microsystems, Germany). Tissue sections (2 - 3 µm) were placed on SuperFrost®Plus positively charged glass slides (Menzel-Gläser, Germany) and dried for 24 h at 40°C. Slides were deparaffinized, rehydrated in graded EtOH and endogenous peroxidase activity was blocked in 1.5% H<sub>2</sub>O<sub>2</sub> in Tris-buffered saline (TBS) for 10 min. De-masking of antibody was performed by heat-induced epitope retrieval (HIER) by boiling slides in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA, pH 9.0) in a microwave oven for 15 min. Non-specific binding of antibodies was blocked with 2% BSA in TBS at rt for 10 min. Antibodies were diluted in 1% BSA in TBS and slides were incubated at 4°C o/n with a monoclonal antibody (1:100) raised against *A. salmonicida* (FM-020AY-5, ibt systems GmbH, Germany). Primary antibody binding was detected with UltraVisionQuanto Detection System HRP Quanto and DAB Quanto (TL-125-QHD, Thermo Scientific, Denmark) with final color development in carbazole solution for 15 min (50 mM acetic acid buffer, pH 5.0 with 4 mg 3-amino-9-ethyl carbazole and 0.015 % H<sub>2</sub>O<sub>2</sub>). All sections were counterstained with Mayer's hematoxylin (Dako, Denmark) for 30 sec and subsequently mounted in water soluble mounting medium Aquamount (Merck, UK). Slides were examined using a Leica DM5000 B microscope (Leica Microsystems, Germany) and micrographs captured by a Leica MC170 HD camera (Leica Microsystems, Germany).

## 2.6. Statistical Analysis

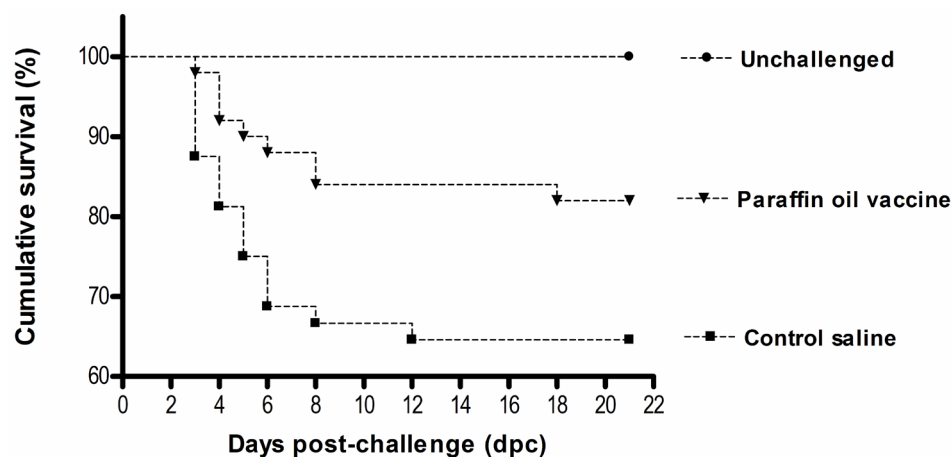
The relative percent of survival (RPS) was calculated at day 21 pc according to Amend [19]:  $RPS = [1 - (\% \text{ mortality in vaccinated} / \% \text{ mortality in saline control})] \times 100$ . All statistical tests were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, USA, [www.graphpad.com](http://www.graphpad.com)) and P-values < 0.05 were considered statistically significant. Mortality data were analyzed using Kaplan-Meier survival analysis and log-rank test. Duplicate groups were pooled after survival curve comparison as they showed no significant difference.

## 3. Results

### 3.1. Challenge Experiments

Mortality commenced at day 3 pc in both vaccinated and sham-vaccinated groups, continued steadily until day 8 pc where after slight mortality occurred until day 21 pc (Figure 2). Fish vaccinated with paraffin oil-adjuvanted bacterin exhibited significantly higher survival (82%, RPS 49) compared to the saline-injected control fish (65%). No mortalities occurred among unchallenged fish.

Moribund fish exhibited disease signs including formation of furuncles, vent and fin base hemorrhages, darkening of the skin and lethargy. Moribund fish showed different degrees of tail fin damage from almost macroscopically non-visible lesions via fin ray splitting to serious inflammation and loss of fin tissue.



**Figure 2.** Survival curves (pooled) of rainbow trout after challenge with *Aeromonas salmonicida* subsp. *salmonicida* (AS). Challenge was conducted by puncturing the epidermis of the dorsal part of the tail fin and placing 100  $\mu$ l of AS strain 090710-1/23 broth culture ( $3.4 \times 10^8$  CFU mL<sup>-1</sup>) on the puncture site for 60 s.

### 3.2. SEM

Tail fins from unchallenged control fish showed a smooth and intact epidermis and revealed no evidence of fin damage (**Figure 3(A)**). The fin samples collected immediately after the challenge (0 day pc) showed considerable damage induced by the multi-puncture device including numerous clefts in the epithelium (**Figure 3(B)**). The tail fins of challenged fish collected at day 3 and 5 pc showed signs of necrosis at the puncture site (**Figure 3(C)**) and bacteria adhering to the fin rays (**Figure 3(D)**). At day 21 pc the puncture area was macroscopically investigated revealing no persisting damage but healed puncture wounds were observed (not shown).

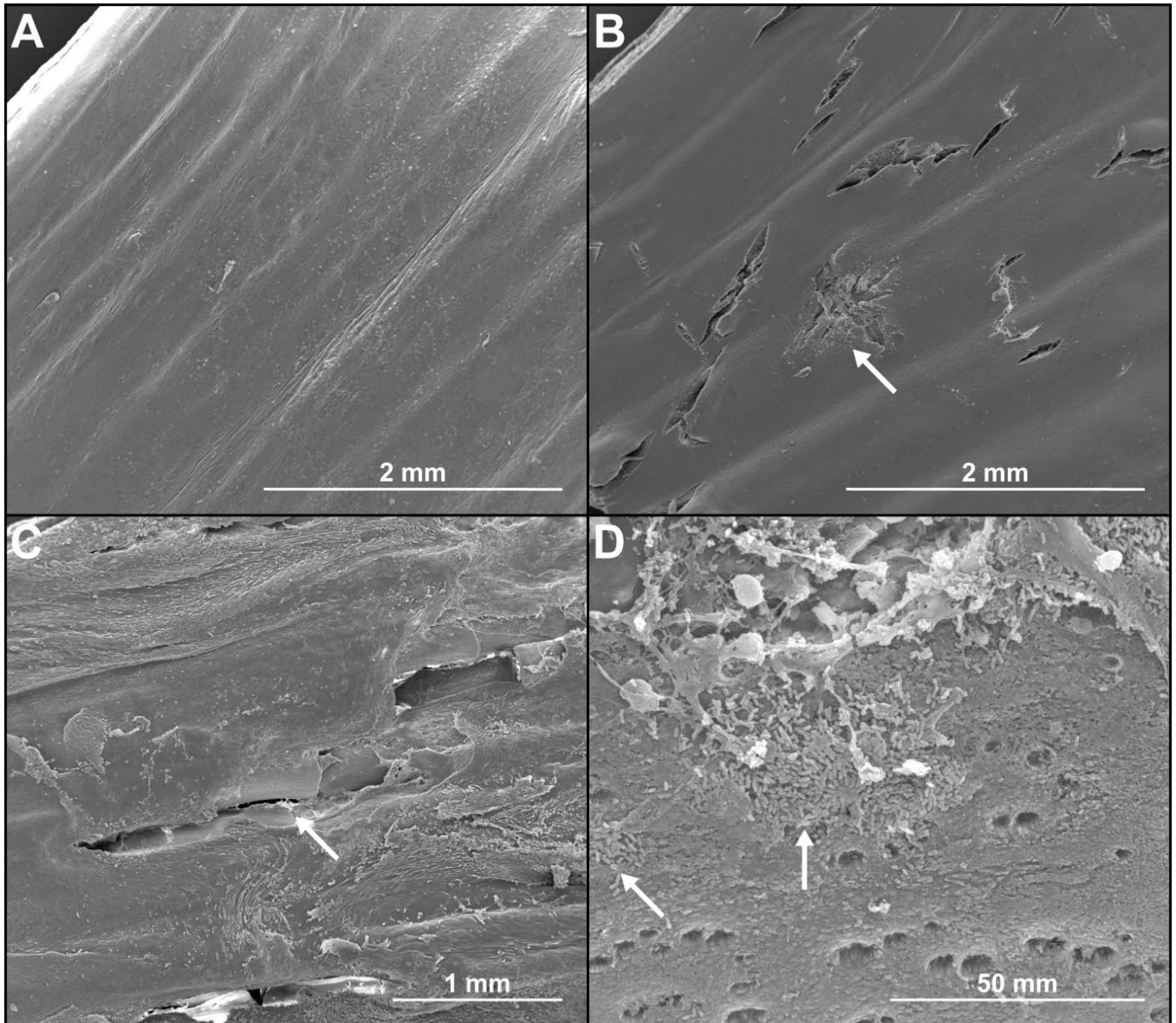
### 3.3. Immunohistochemistry

The immunohistochemical analysis revealed AS bacteria around the puncture site after challenge (**Figure 4(A)**, **Figure 4(B)**). The presence of AS was detected as early as day 0 pc when few bacterial cells were attached to the tail fin epithelium, at day 3 pc AS were found in high concentrations predominantly at the puncture wounds (**Figure 4(A)**) and from day 4 bacteria also colonized the surrounding tissues (**Figure 4(B)**). No bacteria were recorded in the tail fin tissue sections of surviving fish at day 21 pc, but some bacterial cells were found in spleen associated with melanomacrophage centers (**Figure 4(C)**).

## 4. Discussion

The present study describes a new challenge method for infection of rainbow trout with AS causing furunculosis in salmonid fish. It is suitable for studies on efficacies of furunculosis vaccines for rainbow trout. The method was developed based on a hypothesis that tail fin injuries may be one of the natural infection routes of AS in salmonids in aquaculture settings.

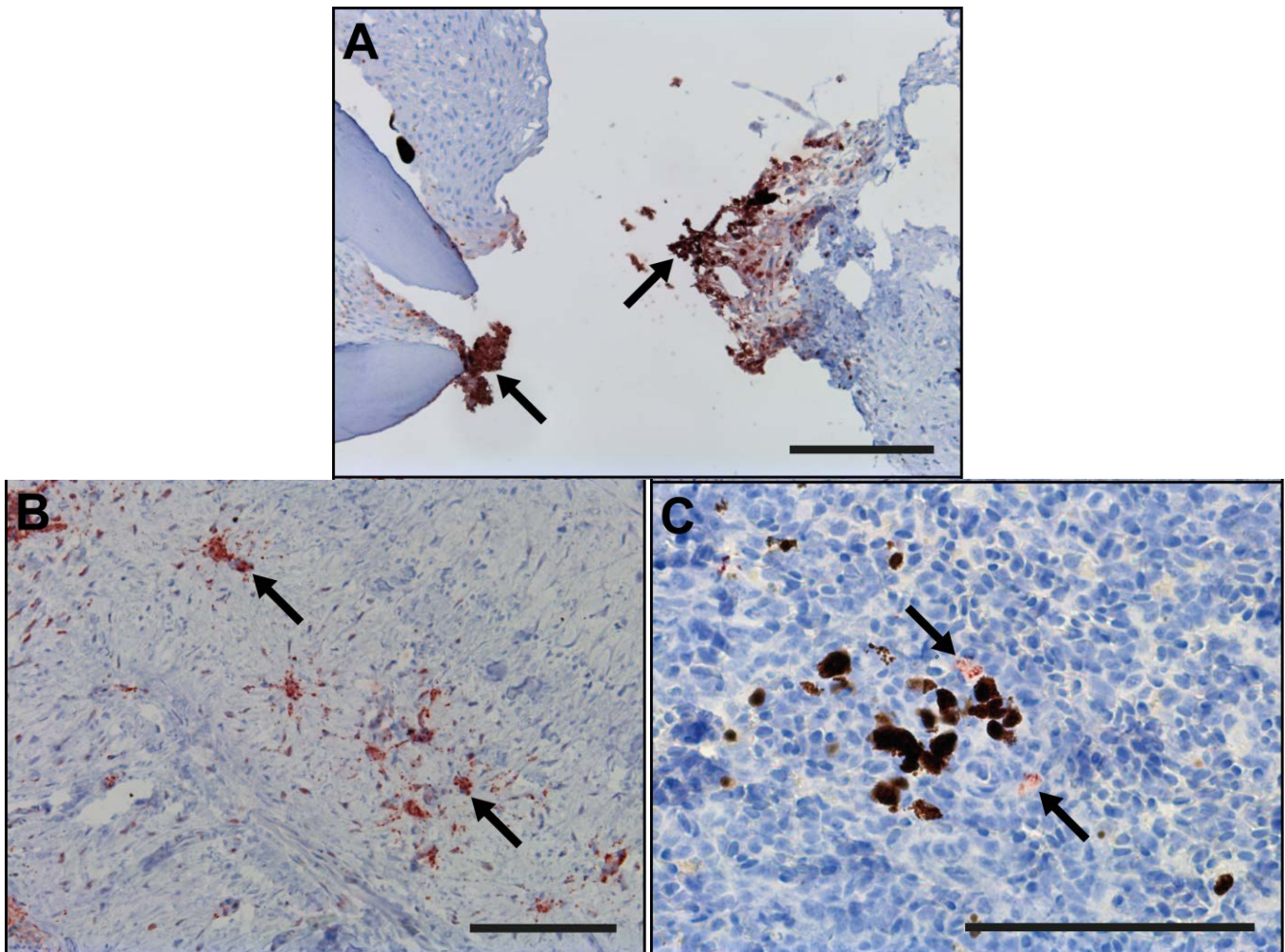




**Figure 3.** Scanning electron microscopy (SEM) images of tail fins after challenge with *Aeromonas salmonicida* subsp. *salmonicida* (AS). (A) The intact fin epithelium of unchallenged fish. (B) The puncture hole (arrow) in fin epithelium of fish immediately after multi-puncture challenge. (C) The puncture site at day 3 post-challenge (pc) shows signs of erosion and loss of epithelium, revealing a fraction of a fin ray (arrow). (D) Bacteria (arrows) adhering to the fin ray at day 3 pc.

Various entry sites for AS have been suggested by different authors and while there's still considerable disagreement about the subject, the gills [20]-[25], the fins [24] [26] and the gastrointestinal track [20] have been suggested as possible entry sites for the bacterium. Salmonid species demonstrate different levels of susceptibility towards AS infection [27] [28]. Thus, rainbow trout is less susceptible compared to East Atlantic salmon and more susceptible compared to Baltic salmon but the underlying mechanisms are unknown. It has been suggested that AS-inhibitory properties in the mucus may counteract infection in rainbow trout [29] [30] but the ability of AS to invade and





**Figure 4.** Immunohistochemistry (IHC) images of tail fins after challenge with *Aeromonas salmonicida* subsp. *salmonicida* (AS) (scale bar 100  $\mu$ m). The presence of AS cells was confirmed by IHC using a monoclonal antibody against *A. salmonicida* (1:100). (A) High concentrations of bacteria (arrows) were observed around the puncture wounds at day 3 post-challenge (pc). (B) Bacteria spread to surrounding tissues forming micro-colonies (arrows) at day 12 pc. (C) Bacterial cells present in spleen associated with melanomacrophage centers at day 21 pc.

survive in undamaged skin and mucus of rainbow trout is still controversial [20] [22] [23] [25] [31] [32]. Skin openings and wounds have been unanimously proposed as infection sites in different salmonid species and some authors [33] managed only to infect rainbow trout with AS in bath challenge after skin abrasion. Similarly, masu salmon bathed in AS bacterium became infected only after injuring the tail and dorsal fins [34] and Svendsen *et al.* [23] showed that a damaged mucus layer and artificial wounds resulted in significantly higher mortality in Atlantic salmon following increased uptake of bacteria in wounded areas [25].

The present infection method aims to mimic the bacterial uptake through skin lesions by resembling the actual infection situation in fish farms where aggressive encounters often results in physical injuries including fin nipping and biting. The problem is well recognized in hatchery-reared salmonids [12] [13] [14] [35] where wounds

may cause fin rot [14] [36] [37] as they predispose fish to secondary bacterial infections including with AS [14] [37] [38].

The results from this study suggest that rainbow trout became infected with AS through the puncture holes in the tail fin. The SEM images from day 3 and day 5 pc. show considerable epithelial damage to the tail fin and reveal exposed fin rays covered with bacteria. Head-kidney swabs of moribund fish and IHC slides showed the initial presence of AS in and around the puncture sites and a subsequent spread to adjacent tissues. The bacteria may disseminate from the infected fins to other organs via circulation and we detected bacterial cells by IHC in the spleen associated with melanomacrophage centers of surviving fish at day 21 pc. The function of these centers in antigen retention for extended period of time have been discussed previously [39] and may reflect immunological clearance in central immune organs.

We suggest that the puncture technique resembles a natural infection route of AS and may induce systemic immune response mechanisms in rainbow trout. We consider this method useful in testing vaccine efficacies and superior to the injection challenges where the bacteria are deposited directly in the body cavity. Ip. challenge can lead to false interpretation of protection due to the non-specific and local inactivation of bacteria at the injection site [6] [8] [9]. The waterborne challenges have been considered as natural routes of infection but are often less potent and less reproducible in inducing mortality even in high bacterial concentrations. It cannot be excluded that fish in laboratory conditions have intact mucus layer and skin hence the artificial setups do not reflect the real situation in the fish farms where fish generally have epithelial injuries [40].

### Future Perspectives

The 35% mortality obtained in the control group was significantly different from mortality in vaccinated group in this study, but it does not meet the recommendations by Amend, 1981 [19]. We therefore prolonged the bacterial exposure time up to 90 sec in our more recent challenge setups and obtained >50% mortality in unvaccinated controls (unpublished data). As behavioral studies in juvenile rainbow trout [12] and Atlantic Salmon Parr [37] have confirmed that the dorsal fin is more prone to bite attacks, the puncture device method could possibly also be used for dorsal fin puncturing.

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